Apical Recycling Endosome-Associated Myosin Vb Is Mutated in Microvillus Inclusion Disease and Is Involved in Intestinal Brush Border Development

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Magdalena R. Golachowska, Agata Szperl, Marcel Bruineberg, Rytis Prekeris, Andy-Mark W.H. Thunnissen, Dick Hoekstra, Cisca Wijmenga, Janusz Ksiazyk, Edmond H. Rings, Martin C. Wapenaar, Sven C. IJzendoorn

Background & Aims: Microvillus inclusion disease is a rare and fatal congenital enteropathy, presenting with intractable secretory diarrhea shortly after birth. The complete inability to absorb nutrients from intestinal lumen demands total parenteral nutrition, and, eventually, transplantation of the small intestine. MID characteristics varies among patients and generally comprises of villous atrophy and crypt hyperplasia, and, at the cellular level, by the apical brush border atrophy, accumulation of apical proteins, lysosomes and microvilli-like inclusions in the apical cytoplasm of intestinal absorptive cells. Previously we have shown that MID enterocytes display abnormal expression of apical recycling endosomal markers, i.e. Rab11a, FIP-1 (RCP), FIP-5 (Rip11), resulting in a defective apical recycling system in MID. In this study, we aimed to identify the genetic cause and functional consequences that underlie the microvillus inclusion disease. Methods: We screened the genomic DNA of three patients diagnosed with microvillus inclusion disease, their siblings and parents. Biopsies of small intestine from MID and control patients were used to analyze the organization of organelles and localization of proteins involved in intracellular trafficking of brush border proteins. Results: In all MID patients together we have identified two substitutions, one deletion, and two protein truncating mutations in the myosin 5B gene. The MYO5B encodes for an actin filament-binding molecular motor protein that interacts with the small GTPase Rab11a, a marker of recycling endosomes, and thus facilitates the intracellular trafficking of apical proteins towards the apical membrane. We also found aberrant expression and subcellular distribution of myosin Vb protein and other key proteins that interact with myosin Vb and/or control apical recycling endosome-mediated protein trafficking. Conclusions: The endosomal system that ensures the recycling of brush border proteins, with myosin Vb as a critical regulator, is required to develop and maintain functional apical cell surface in human enterocytes, and perturbations in this can be causally linked to microvillus inclusion disease. Mutations occurring at different positions of MYO5B gene and thus affecting different functional regions of MYO5B protein could explain the diversity of phenotypes present in MID patients. The identification of mutations in MYO5B as the cause for MID brings a major advance in setting the reliable diagnosis, enables the genetic counseling and prenatal screening, as well as paves the way for developing alternative therapeutic strategies.

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Intestine Specific Deletion of N-WASP Leads to Alteration of Gut Homeostasis in Mice

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Background: Wiskott-Aldrich Syndrome protein (WASP) is a cytoplasmic protein in hematopoietic cells that regulates actin assembly via the Arp2/3 complex. Deficiencies in WASP are associated with IBD in humans and spontaneous colitis in mice, and recent genome-wide linkage studies provide evidence of novel susceptibility factors for UC in the region of the Arp2/3 locus. We sought to determine the role of the ubiquitously expressed N-WASP protein, a key regulator of Arp2/3 activity and cytoskeletal dynamics, in the intestinal epithelium. **Methods:** To generate mice with gut-restricted deletion of N-WASP (intestine NWASP KO, iNWKO), mice expressing Cre recombinase under the control of the villin promoter ($tg^{v\text{-}cre}$) were mated to mice homozygous for a floxed N-WASP allele ($N\text{-}WASP^{L2L/L2L}$). In all experiments, iNWKO mice ($N\text{-}WASP^{L2L/L2L}tg^{v\text{-}cre}$) were compared with $N\text{-}WASP^{L2L/L2L}$ lacking the villin-Cre transgene. Intestinal epithelial cells were isolated by EDTA dissociation and centrifugation, and tissue was examined with H&E, Alcian blue, immunofluorescence and electron microscopy (EM). To examine proliferation and migration of N-WASP deficient enterocytes, mice were injected IP with BrdU and sacrificed at 2 and 24 hours. To assess N-WASP deletion in inflammation, 6-week old mice (5 KO,5 WT) were given 3.5% DSS in drinking water for 5 days, and sacrificed on day 12 for histology. **Results:** iNWKO mice were viable and fertile, but failed to appropriately gain weight; adults weighed on average 30% less than their WT ($N\text{-}WASP^{L2L/L2L}$) littermate controls (n=25,p=0.007). There was no spontaneous development of enterocolitis. N-WASP deletion was confirmed by PCR, and the absence of protein confirmed by Western blot on intestinal epithelial lysates. Numbers of Paneth, goblet and BrdU-incorporating crypt progenitor cells were similar. Notably, epithelial cells in iNWKO mice contained uncondensed nuclei and there was markedly increased enterocyte migration at 24 hours. EM revealed disorganized and clustered colonic microvilli and the absence of a terminal web. The distribution of E-cadherin was similar between iNWKO mice and WT controls. iNWKO mice given DSS exhibited more weight loss, and a trend toward higher clinical scores of disease activity. Histology was similar in both DSS groups, with the exception of one iNWKO mouse that required euthanasia on day 9; this mouse demonstrated severe architectural changes, ulceration, and neutrophilic infiltrate. **Conclusion:** Deletion of intestinal N-WASP leads to a phenotype of wasting, increased intestinal cell turnover and microvillus structural abnormalities, linking the actin cytoskeleton to the maintenance of gut homeostasis.

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The Role of MicroRNAs in the Intestinal Epithelium

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MicroRNAs (miRNA) are small single stranded RNA molecules, approximately 22 nucleotides in length, that function in fine-tuning gene expression. miRNAs are processed from primary transcripts by enzymes including Drosha and Dicer, which are essential for production of functional miRNAs. We tested the role miRNAs play in the development and function of the intestinal epithelium, using a conditional knockout strategy in which the Dicer enzyme was deleted starting at embryonic day 12.5, effectively eliminating all mature miRNAs in this tissue. DicerL/LVillinCre mutants and controls are born at the same weight; however, the mutants fail to grow at the same rate as their littermate controls, with a portion dying before weaning at p21. DicerL/LVillinCre pups are not only smaller but also dehydrated, with severely loose stool containing large amounts of fat, suggesting compromised lipid absorption in the small intestine. Once weaned and placed on traditional mouse chow, surviving mutants catch up in weight to their littermates; however, they still have a higher fecal water content than the controls, suggesting impaired colonic function. When placed on a high fat diet, a stress to the gastrointestinal tract, again the mutants' stool contains large amounts of fat that is not seen in the control's stool. At 8 weeks, the gastrointestinal tract displays noticeable structural differences between mutants and controls. There is a marked decrease in mucus producing goblet cells in the colon of Dicer deficient mice, as well as an anteriorization of the colon seen by the expression of alkaline phosphatase along the borders of the crypts and a decrease in Cdx2 expression. As the expression pattern of miRNAs in the intestinal epithelium is not known, we have determined the miRNA profile of the intestinal epithelium using genome-wide highthroughput sequencing. While some known miRNA have been identified, there is also evidence that novel miRNAs are expressed in the intestinal epithelium. Current studies include further analysis of the fat absorption and digestion pathway in these mutants, as well as histological studies on pre-weaned pups.

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The ZO-1 Actin Binding Region (ABR) Is Required for Cytoskeletal Tight Junction Regulation

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In inflammatory bowel disease, myosin light chain kinase (MLCK) is a critical mediator of actomyosin-dependent barrier regulation. However the molecular mechanisms involved are poorly understood. ZO-1, which binds directly to actin via the ABR, links actin to other tight junction proteins and may participate in barrier regulation. The aim of this study is to determine the role of the ZO-1 ABR in MLCK-dependent tight junction regulation. **METHODS:** Fluorescent-tagged proteins were expressed in Caco-2 monolayers and transgenic mice. Dynamic behavior was assessed by fluorescence recovery after photobleaching (FRAP). Monolayers and mice were treated with PIK, a membrane Permeant Inhibitor of MLC Kinase, as indicated. **RESULTS:** MLCK inhibition enhances the tight junction barrier *In Vivo* and *In Vitro*. In Caco-2 monolayers, PIK does not affect dynamic behavior of actin, occludin, or claudin-1, but does increase the ZO-1 immobile fraction from 45% to 80%. *In Vivo*, epithelial ZO-1 exchange between cytosolic and tight junction pools was retarded in jejunal mucosa of MLCK knockout mice and accelerated by constitutively-active MLCK expression. Moreover, PIK stabilized ZO-1 in wild type, but not MLCK knockout, mice. Thus, MLCK-dependent regulation of ZO-1 stability occurs *In Vivo* and ZO-1 stabilization by PIK requires MLCK. To define mechanisms of ZO-1 stabilization, a deletion mutant lacking the ABR, ZO-1ΔABR, was expressed in Caco-2 cells. Only 27% of ZO-1ΔABR is immobile and this fraction is not increased by MLCK inhibition. The ABR is, therefore, necessary for both basal and regulated ZO-1 stabilization. However, the ABR must cooperate with other domains, as free ABR is completely mobile, is not stabilized by MLCK inhibition, and suppresses normal ZO-1 dynamic behavior at the tight junction. To determine if free ABR or ZO-1ΔABR are able to suppress barrier enhancement induced by MLCK inhibition, the effect of PIK was compared in Caco-2 monolayers. PIK causes a dose-dependent increase in transepithelial resistance in nontransfected monolayers and those transfected with full length ZO-1. In contrast, the effect of PIK is muted by ZO-1ΔABR expression and almost completely blocked by free ABR expression. Thus, barrier function increases after MLCK inhibition require direct interactions between ZO-1 and actin. **CONCLUSION:** The ABR participates in ZO-1 dynamic behavior at the tight junction and is required for ZO-1 stabilization after MLCK inhibition. The effects of free ABR and ZO-1ΔABR on ZO-1 dynamic behavior and their dominant negative effect on barrier enhancement by MLCK inhibition suggest that the ZO-1 ABR is critical to cytoskeletal tight junction regulation.

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Notch1 Activation Promotes Goblet Cell Depletion and Expression of Pla2g2a in the Inflamed Mucosa of Ulcerative Colitis

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Background & Aims: Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease, characterized by inflammation and ulcer formation of the colonic mucosa. Besides infiltration of inflammatory cells in the lamina propria, "goblet cell depletion" and increased expression of Paneth cell specific genes, such as PLA2G2A, have been frequently observed in the epithelia of UC patients. However, the molecular mechanism mediating such response of the epithelia has never been described. The aim of this study was to elucidate the role of Notch signaling pathway in pathophysiology of UC. Methods: Immunostaining for cleaved Notch1 (NICD1) was performed to determine expression and activation of Notch1 within the human intestinal mucosa. A γ-secretase inhibitor was used to inhibit endogenous activation of Notch, whereas Tetracycline-dependent expression system was used for over-expression of NICD1, within human colonic epithelial cell-lines. Change in gene expression upon such up- or down-regulation of intracellular Notch activation in intestinal epithelial cells (IECs) was determined by quantitative RT-PCR, and further confirmed by immunostaining or ELISA assay. Results: Immunostaining of normal small intestinal or colonic mucosa revealed activation of Notch1 in IECs residing at the lower part of the crypt. Double immunostaining showed that activation of Notch1 was completely absent in goblet cells, but in sharp contrast, clearly present in Paneth cells. Consistently, inhibition of endogenous Notch activation significantly promoted goblet cell differentiation of HT29 and LS174T cells, as shown by increase of MUC2 expression. Conversely, forced expression of NICD1 significantly suppressed MUC2 expression in LS174T cells, resulting in depletion of goblet cells *In Vitro*. In addition, both quantitative RT-PCR and ELISA assay revealed significant up-regulation of PLA2G2A secretion upon Notch1 activation in LS174T cells. Such effect of Notch1 activation in IECs appeared to be present in the inflamed mucosa of UC, as our immunohistochemical analysis showed increased number of Notch1-activated IECs in goblet cell depleted crypts, and clear activation of Notch1 in IECs showing ectopic expression of PLA2G2A. Conclusion: Activation of Notch1 in IECs promotes both goblet cell depletion and expression of PLA2G2A